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Isolation and characterization of a thermostable F_{420}:NADPH oxidoreductase from *Thermobifida fusca*

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Running title: F_{420}:NADPH oxidoreductase from *T. fusca*

Abbreviations used: FNO, F_{420}:NADPH oxidoreductase; Tfu, *Thermobifida fusca*; Af, *Archaeoglobus fulgidus*; NAD(P)$^+$, nicotinamide adenine dinucleotide (phosphate)

Abstract

$F_{420}H_2$-dependent enzymes reduce a wide range of substrates that are otherwise recalcitrant to enzyme-catalyzed reduction, and their potential for applications in biocatalysis has attracted increasingly attention. *Thermobifida fusca* is a moderately thermophilic bacterium and holds high biocatalytic potential as a source for several highly thermostable enzymes. We report here on the isolation and characterization of a thermostable F_{420}:NADPH oxidoreductase (Tfu-FNO) from *T. fusca*, being the first F_{420}-dependent enzyme described from this bacterium. Tfu-FNO was heterologously expressed in *Escherichia coli*, yielding up to 200 mg recombinant enzyme per liter of culture. We found that Tfu-FNO is highly thermostable, reaching its highest activity at 65 °C and that Tfu-FNO is likely to act *in vivo* as an F_{420} reductase at the expense of NADPH, similar to its counterpart in *Streptomyces griseus*. We obtained the crystal structure of FNO in complex with NADP$^+$ at 1.8 Å resolution, providing the first bacterial FNO structure. The overall architecture and NADP$^+$-binding site of Tfu-FNO were highly similar to those of the *Archaeoglobus fulgidus* FNO (Af-FNO). The active site is located in a hydrophobic pocket between an N-terminal dinucleotide-binding domain and a smaller C-terminal domain. Residues interacting with the 2′-phosphate of NADP$^+$ were probed by targeted mutagenesis, indicating that Thr28, Ser50, Arg51, and Arg55 are important for discriminating between NADP$^+$ and NAD$^+$. Interestingly, a T28A mutant increased the kinetic efficiency more than three-fold as compared with the wild-type enzyme when NADH is the substrate. The biochemical and structural data presented here provide crucial insights into the molecular recognition of the two cofactors, F_{420} and NAD(P)H by FNO.

Introduction

Flavins can arguably be regarded as the most extensively studied redox cofactors. One natural flavin analogue is cofactor F_{420} which was first isolated and characterized from methanogenic archaea in 1972 (1). Since then, F_{420} has been found in members of methanogens, actinomycetes, cyanobacteria, and some betaproteobacteria (2). Replacement of the 5′ nitrogen of flavins with a carbon in F_{420}, resulting in a so-called deazaflavin, renders the cofactor nearly unreactive towards molecular oxygen. Hence, F_{420} is an obligate hydride-transfer cofactor similar to the nicotinamide cofactors (Fig. 1). Besides, the 8′-OH group on the isoalloxazine ring in

F_{420} has been suggested to slow down the autooxidation of the reduced cofactor ($F_{420}H_2$) in air, thus the reduced species is much more stable than that of flavins (3).

Many $F_{420}(H_2)$-dependent enzymes have been characterized recently and their potential for applications in biocatalysis has attracted increasing attention (4,5). F_{420}-dependent enzymes studied so far have been shown to be capable of reducing a wide range of substrates which are otherwise recalcitrant to enzyme-catalyzed reduction (4,5). However, the commercial unavailability of cofactor F_{420} remains a bottleneck for studying and applying the respective enzymes. Therefore, it would be attractive to have access to an efficient $F_{420}H_2$ cofactor recycling system. In this context, F_{420}:NADPH oxidoreductases (FNOs, E.C. 1.5.1.40, Fig. 1) could become very valuable as NADPH-driven $F_{420}H_2$-recycling systems. FNOs catalyze the reduction of $NADP^+$ using $F_{420}H_2$ and have been found in a number of archaea (6–10) and bacteria (11) (Fig. 1). It has been argued that in methanogens, FNO catalyzes mainly the reduction of $NADP^+$ using $F_{420}H_2$ while bacterial FNOs are supposed to catalyze the reverse reaction (11).

Thermobifida fusca is a moderately thermophilic soil bacterium with high G+C content. This actinomycete holds high biocatalytic potential as it has already served as a source for several highly thermostable enzymes, e.g., catalase, Baeyer–Villiger monooxygenase, and glycoside hydrolases (12–14). Interestingly, a recent bioinformatic study predicted that the *T. fusca* genome contains 16 genes encoding for F_{420}-dependent enzymes (15). Nevertheless, there has been so far no biochemical evidence for such enzymes. Here, we describe the identification and characterization of a dimeric thermostable F_{420}:NADPH oxidoreductase from *T. fusca* (Tfu-FNO), confirming the presence of F_{420}-dependent enzymes in this mesophilic bacterium. Despite the high GC content (67%) of the gene sequence, Tfu-FNO is readily expressed in *E. coli*. Notably, Tfu-FNO is a thermostable enzyme and shows a clear substrate preference towards NADP(H) instead of NAD(H). By solving the three-dimensional crystal structure of Tfu-FNO, we set out site-directed mutagenesis to corroborate the role of residues that interact with the phosphate moiety at 2′ position of $NADP^+$.

Results

Purification of Tfu-FNO

A BLAST search for Af-FNO homologs in *T. fusca* resulted in the identification of the Tfu_0907 gene (TFU_RS04835). The encoded protein shares 40% and 70% sequence identity to FNOs from *A. fulgidus* and *S. griseus*, respectively (Fig. 2). The Tfu-*fno* gene, with a high GC content (67%), was amplified from the genomic DNA of *T. fusca* and transformed into *E. coli* TOP10 as a pBAD-*fno* construct. Purification of the respective protein, Tfu-FNO, was achieved through ammonium sulfate precipitation followed by anion exchange chromatography. DNase I treatment during the first steps of protein purification was found to be essential to remove residual DNA. Tfu-FNO was obtained in pure form with a relatively high yield: 120–200 mg/L culture. It is worth noting that the amount of purified Tfu-FNO obtained in our system is significantly higher than that of Af-FNO when heterologously expressed in *E. coli* [2 mg/L culture (16)].

Effects of pH and temperature on activity

FNOs are known to catalyze the reduction of $NADP^+$ at higher pH, while at lower pH it catalyzes the reverse reaction. Figure 3 shows the effect of pH on Tfu-FNO activity. The reduction rate of $NADP^+$ is highest at pH 8.5–9.0 while the reverse reaction is optimal between pH 4.0–6.0. From the k_{obs} values of both the forward and backward reactions, it can be concluded that FNO catalyzes $NADP^+$ reduction more efficiently (Fig. 3). This is in line with the redox potential of F_{420} (−340 mV) being lower when compared with that of $NADP^+$ (−320 mV) (3).

Since FNO originates from the mesophilic organism *T. fusca*, the enzyme is expected to be stable at relatively high temperatures. Measuring the activities at temperatures between 25 and 90 °C revealed that the enzyme displays highest activity between 60–70 °C (Fig. 4). The activity at 65 °C is almost 4-time higher than that at 25 °C. The apparent melting temperature of Tfu-FNO was found to be 75 °C, as measured by the Thermofluor® method (17). All the generated Tfu-FNO mutants had melting temperatures similar to the wild-type enzyme (data not shown). This indicates that FNO is remarkably thermostable and is most active at elevated temperatures.

Steady-state kinetics

The steady-state kinetic parameters were measured for NADPH and F_{420} as substrates by following absorbance of these two cofactors at either 340 nm or 400 nm, respectively. The concentration of one substrate was varied while keeping the other substrate at a constant, saturated concentration. The kinetic data fitted well to the Michaelis–Menten kinetic model when the observed rates (k_{obs}) were plotted against substrate concentrations. Tfu-FNO has a K_m value of 7.3 μM and 2.0 μM for NADPH and F_{420}, respectively at pH 6.0 and 25 °C (Table 1). Thus, Tfu-FNO has a significantly lower K_m for NADPH (2.0 μM) compared to the values featured by Af-FNO (40 μM) and FNO from *S. griseus* (19.5 μM) (8,11). The k_{cat} (3.3 s^{-1}) of Tfu-FNO is somewhat lower when compared with that of Af-FNO (5.27 s^{-1}) (18).

The overall structure of Tfu-FNO

Crystallization of Tfu-FNO was successful which allowed the elucidation of its crystal structure. This revealed that $NADP^+$ had been co-purified with the native enzyme as it was found to be bound in the active site (Fig. 5–6). All crystal soaking attempts to obtain the F_{420} cofactor bound in the enzyme active site failed, which can be explained by the tight molecular packing found in Tfu-FNO crystals that would hamper cofactor binding in the same position as found in Af-FNO (Fig. 5A). It is known that, depending on the bacterial species, the number of glutamate moieties of F_{420} can vary from two to nine, with five to six being the predominant species in mycobacteria (19). Given the crystal arrangement of Tfu-FNO molecules, an oligoglutamate tail of F_{420} of any length would clash against another subunit interacting through crystal packing (Fig. 5A). Nevertheless, the architecture of the active site is highly conserved, and $NADP^+$ adopts a virtually identical position with respect to that observed in Af-FNO (Fig. 5B). Therefore, F_{420} was tentatively modelled in Tfu-FNO upon superposition of the archaeal enzyme (Fig. 5C). The modelled F_{420} fits very well into the Tfu-FNO active site without any clashes. Similarly to Af-FNO, F_{420} would bind in Tfu-FNO at the C-terminal domain with its deazaisoalloxazine ring burying deep inside the catalytic pocket and the highly polar oligoglutamyl tail directed towards the exterior of the dimer (Fig. 5B,C).

As mentioned above, $NADP^+$ binds to the N-terminal part of Tfu-FNO in a highly similar manner to that of Af-FNO which is characteristic for members of the dinucleotide binding protein family (20,21). The hydrogen bonding network between $NADP^+$ and the residues that form the active site are illustrated in Fig. 6. In particular, the nicotinamide ring directly docks to the protein by hydrogen bonding the cofactor amide group to the peptide nitrogen of Ala155 (corresponding to Ala137 in Af-FNO). This conserved interaction is believed to be crucial in conferring the *trans* conformation of the amide group. With this conformation, the pyridine ring of $NADP^+$ is maintained planar which in turn facilitates the hydride transfer between the C4 of the NADP and C5 of F_{420} by shortening the distance of the two atoms (20).

$NADP^+$ binding site

The residues involved in binding the ADP moiety are also conserved in Tfu-FNO (Fig. 6). Analogous to Af-FNO, the negatively charged group of the ribose 2′-phosphate interacts with the side chains of Thr28, Ser50, Arg51, and Arg55 (corresponding to Thr9, Ser31, Arg32, and Lys36 in Af-FNO). These residues are highly conserved in other known FNOs (Fig. 2). These residues therefore appear to be crucial for substrate recognition and help to discriminate between $NADP^+$ and NAD^+ (20). To get more insights into the role of these residues, they were mutated into amino acids with different charge and/or size, and tested for the cofactor specificity towards the two nicotinamide cofactors. Table 1 shows the kinetic parameters for both NADH and NADPH as substrate. For wild-type Tfu-FNO, the K_m value for NADH (14 mM) is several orders of magnitude higher than that for NADPH (7.3 μM), clearly confirming that the enzyme prefers NADP(H) over NAD(H). For all mutants, the K_m value for NADPH significantly increased (from 2.6- to >68-fold) compared to that of the wild-type enzyme, which verified the crucial role of these residues in binding NADP(H). Intriguingly, recognition of NADH remained the same or improved in all mutants (Table 3), with a K_m value ranging from 0.23- to 2.3-times of that from the wild-type. Noticeably, R55N and R55S variants have a significantly improved affinity towards NADH. In case of mutant R55N, $K_{m,\ NADPH}$ increased more than 100 fold while the $K_{m,\ NADH}$ decreased almost 4 fold. The S50E mutant was the best among the tested mutants with a $K_{m,\ NADH}$ of almost 5-time lower and a $K_{m,}$

$_{NADPH}$ of approximately 100-fold higher as compared to wild-type Tfu-FNO. Interestingly, the T28A mutant showed an increased activity towards both NADPH and NADH, with a 4-fold increase in catalytic rate (k_{cat} = 14 s^{-1}) for NADPH and a 2.8-fold decrease in K_m value (5 mM) for NADH when compared with the wild-type enzyme. This resulted in significantly improved k_{cat}/K_m values for both NADPH and NADH, respectively. Unfortunately, combinations of the mutations did not show significant additive effects (Table 1).

Discussion

F_{420}-dependent enzymes are interesting candidates for biotechnological applications (5). Recent studies have suggested a widespread occurrence of such deazaflavin-dependent enzymes in actinobacteria (15). Some specific lineages seem especially rich in F_{420}-dependent enzymes, such as *Mycobacterium tuberculosis*. This makes members of this superfamily of deazaflavoproteins potential drug targets due to their absence in the human proteome and the human gut flora. The work of Selengut et al. also predicted the presence of at least 16 F_{420} related genes in *T. fusca,* including all genes required for F_{420} biosynthesis (15). Through our study, we have experimentally confirmed the presence of an F_{420}-dependent enzyme in this actinomycete by cloning and characterization of a thermostable F_{420}:NADPH oxidoreductase (Tfu-FNO), which catalyzes the reduction of $NADP^+$ using reduced F_{420} and the reverse reaction.

The role of FNO in generating reduced F_{420}

F_{420} cofactor provides microorganism alternative redox pathways. The deazaflavin cofactor seems especially equipped for reduction reactions as it displays a redox potential which is lower when compared with the nicotinamide cofactor. Two enzymes have been identified in previous studies that serve a role in reducing F_{420}—FNO and F_{420}-dependent glucose-6-phosphate dehydrogenase (FGD) (4). Using *T. fusca* cell-free extract and heterologously expressed Tfu_1669 (a putative *M. tuberculosis* FGD homolog), we could not detect any FGD activity (unpublished data). This suggests that the *T. fusca* proteome indeed does not include an FGD. In fact, it has been shown before that not all actinomycetes have an FGD (22). Therefore, FNO may be the primary enzyme in actinomycetes for providing the cells with $F_{420}H_2$. Nevertheless, at physiological pH (7.0–8.0, Fig. 3) Tfu-FNO performs reduction of $NADP^+$ slightly better than reduction of cofactor $F_{420,}$ which is different from the FNO from *S. griseus* (11) and more similar to the archaeal FNOs (7,8). This can partly be explained by the experimental condition (24 °C) differing from the optimum temperature at which the bacteria grow (55 °C) and the intercellular environment (e.g., cofactor concentrations, salt concentrations). Several lines of evidence suggest that in other actinomycetes, such as *Rhodococcus opacus* and *Nocardioides simplex*, FNO is also the main source of $F_{420}H_2$. In these bacteria, the *fno* gene was embedded in the same operon with genes encoding for the $F_{420}H_2$-dependent reductases, which are involved in the metabolism of picrate and 2,4-dinitrophenols (23–25). FNO-catalyzed regeneration of $F_{420}H_2$ was also proposed to be crucial for the reductive steps in the biosynthesis of tetracycline by *Streptomyces* (26).

Structure and NADP(H) binding site of Tfu-FNO

FNO is believed to be the only F_{420}-dependent enzyme known so far that is conserved between archaea and bacteria (4). Except for a 19 amino acid extension loop at the N-terminus, Tfu-FNO largely shares the overall topology and cofactor binding site with that from *A. fulgidus* (Fig. 2 and 5B). The residues that interact directly with the 2′-phosphate group of NADP(H) are also highly conserved (Fig. 6), and have proven to be essential for binding this cofactor. Upon disrupting the hydrogen bonding network by mutagenesis, all the mutants lost virtually all ability to recognize NADPH (Table 1). Intriguingly, the affinity of these variants towards NADH improved, with the T28A mutant being the best in terms of specificity for NADH (3.3-fold higher k_{cat}/K_m than that of WT). Yet, an efficient NADH-dependent FNO has still to be engineered. For this, a newly developed tool could be explored which can guide structure-inspired switching of coenzyme specificity (27).

Potential applications in biocatalysis

Tfu-FNO represents a highly attractive candidate for the biocatalytic reduction of F_{420}. The enzyme is very thermostable, remains active over a wide range of pH (Fig. 3,4), and can be easily expressed in *E. coli* (120–200 mg/ L culture). Tfu-FNO is also a relatively fast enzyme, especially with the T28A mutant displaying a k_{cat} of 14 s^{-1} for NADPH (Table 1). Whereas the majority of current enzymatic $F_{420}H_2$ regeneration protocols employ FGDs (28,29), the cost of the expensive, non-recyclable cosubstrate glucose-6-phosphate remains the main bottleneck for the use of such enzyme in large-scale

applications. Therefore, an $F_{420}H_2$-generating system whose cosubstrate could be recycled, such as T28A TfuFNO would be highly promising. Available, robust NAD(P)H regeneration machineries, such as glucose dehydrogenase or other dehydrogenases, have been thoroughly investigated and widely applied in industry (30). Therefore, by combining Tfu-FNO with an appropriate NAD(P)H recycler, $F_{420}H_2$-reductases can be exploited for biocatalytic purposes.

Experimental procedures

Cloning, expression, and purification of Tfu-FNO

Thermobifida fusca YX was grown at 55 °C in Hägerdahl medium and its genomic DNA was extracted using the GeneElute Bacterial Genomic DNA kit (Sigma–Aldrich). The gene Tfu-*fno* (Tfu_0970, TFU_RS04835) was PCR amplified from genomic DNA of *T. fusca* using the pair of primers listed in Table 2 with the *Nde*I and *Hind*III restriction sites introduced at the 5′ and 3′ positions of the gene, respectively. The purified PCR product and the pBAD*N*/*Myc*-HisA vector were digested with the restriction enzymes *Nde*I and *Hind*III, purified, and ligated [vector to insert ratio ca. 1 to 5 (mol/mol)] using T4 DNA ligase (Promega) with quick ligation buffer. The pBAD*N*/*Myc*-HisA vector is a variant of the commercial pBAD/*Myc*-HisA (Invitrogen) where the unique *Nco*I site at the translation start is replaced with *Nde*I. The ligation product was transformed into chemically competent *E. coli* TOP10 cells using the heat shock method. Correct transformants were confirmed by sequencing the recombinant plasmid pBAD-*fno*.

Site-directed mutagenesis was carried out by using the pBAD-*fno* vector as template and the QuikChange® mutagenesis method with the corresponding pairs of primers listed in Table 2. The primers (200 nM) were used in a 10 µL reaction mixture. In case of the double mutants, plasmids with a single mutation were used as template. The remaining parent template vector was digested by incubating with *Dpn*I (New England Biolab) at 37 °C for 2 h. *Dpn*I was then inactivated at 80 °C for 10 min and the mutant plasmid was transformed into chemically competent *E. coli* TOP10 cells. Mutations were confirmed by sequencing.

E. coli TOP10 cells with pBAD-*fno* were grown overnight at 37 °C, 130 rpm in a 5 ml lysogenic broth (LB) containing 50 µg/ml ampicillin. This pre-culture was used to inoculate 500 ml of the same medium and grown at 37 °C, 130 rpm. When the OD_{600} reached 0.4–0.6, the protein expression was induced by addition of 0.02% (w/v) arabinose followed by incubation at 30 °C, 130 rpm for 12 h. Cells were harvested by centrifugation at 6000 × g for 15 min (JLA 10.500 rotor, 4 °C) and resuspended in 10 ml of 50 mM KPi pH 7.0 supplemented with 1 µg/ml of DNase I. Cells were sonicated for 7 min (10 sec on, 15 sec off cycle, 70% amplitude) at 4 °C using a VCX130 Vibra-Cell sonicator (Sonics & Materials, Inc., Newton, USA), and then centrifuged at 15000 × g (JA 17 rotor) for 45 min to obtain the cell-free extract. Tfu-FNO was precipitated by adding 50% saturated ammonium sulfate followed by anion exchange chromatography with a HiTrap™ Q HP 5ml (GE Healthcare) column pre-equilibrated with the same resuspension buffer. Tfu-FNO was eluted by using a linear gradient of 0–1 M NaCl in the same buffer. At around 250 mM NaCl, Tfu-FNO started eluting. Excess salt was removed by using PD-10 desalting column and the protein was stored in 50 mM KPi buffer. (GE Healthcare). Protein concentration was estimated using Bradford assay (30).

Temperature, pH optima, and thermostability of Tfu-FNO

F_{420} was isolated from *Mycobacterium smegmatis* mc^2 4517 as previously published protocol (32). $F_{420}H_2$ was prepared by biocatalytic reduction of F_{420} using a recombinant F_{420}-dependent glucose-6-phosphate dehydrogenase from *Rhodococcus jostii* RHA1 (29) as previously described (28).

The apparent melting temperature, T_m, was determined using the *Thermoflour*® technique (17) with a Bio-Rad C1000 Touch Thermal Cycler (Bio-rad Laboratories, Inc.). The reaction volume was 25 µL, containing 10 µM of enzyme and 5 µL of 5 × SYPRO Orange (Invitrogen). To determine the temperature for optimal activity of Tfu-FNO, the enzyme activity was measured using 1.25 mM NADPH and 20 µM F_{420} in 50 mM KPi pH 6.0 in a 100 µL reaction volume. The cuvette containing the substrates in preheated buffer was heated to the tested temperature (25–90 °C) and the reaction was started by adding 10 nM enzyme. The pH optimum was determined for both the forward and backward reactions of Tfu-FNO. F_{420} depletion at 400 nm (ε_{400} = 25 $mM^{-1}\,cm^{-1}$) (33,34) or NADH formation at 340 nm (ε_{340} = 6.22 $mM^{-1}\,cm^{-1}$) was followed using a V-660 spectrophotometer from Jasco (IJsselstein, The Netherlands). In this experiment, the reaction (100 µL) contained 250 µM NADPH and 20 µM F_{420} (F_{420} reduction); or 250 µM $NADP^+$ and 20 µM

$F_{420}H_2$ ($NADP^+$reduction) in 50 mM buffer. Sodium acetate, KPi and Tricine-KOH based buffers were used for pH 4.5–5.5, 6.0–7.5 and 8.0–9.5, respectively.

Steady-state kinetic analyses

To determine the kinetic parameters of the enzyme, initial F_{420} reduction rates were measured using a SynergyMX microplate reader (BioTek) using 96-well F-bottom plates (Greiner Bio-One GmbH) at 25 °C. The reaction was performed in 50 mM KPi pH 6.0 and was started by adding 25–50 nM enzyme in the final volume of 200 µL. The concentration of one of the substrates was kept constant (250 µM for NADPH and 20 µM for F_{420}, respectively) while varying the concentration of the other substrate. All the measurements were performed in duplicate. A decrease of absorption either at 400 nm (F_{420} reduction, ε_{400} = 25.7 $mmol^{-1}$ cm^{-1}) or at 340 nm (NADPH oxidation, ε_{340} = 6.22 $mmol^{-1}$ cm^{-1}) was followed to determine the observed rates, k_{obs} (s^{-1}). K_m and k_{cat} values for $NADP^+$, NADPH, F_{420} and $F_{420}H_2$ were calculated by fitting the data into the Michaelis–Menten kinetic model using nonlinear regression with GraphPad Prism 6.00 (GraphPad Software, La Jolla, CA, USA).

Crystallization, X-ray data collection, and structure determination of Tfu-FNO

Native Tfu-FNO was crystallized using the sitting-drop vapour diffusion technique at 20 °C by mixing equal volumes of 9.0 mg/mL protein in 10 mM Tris/HCl pH 7.5, 100 mM NaCl and of the reservoir solution containing 5% (w/v) PEG 3000, 30% (v/v) PEG 400, 10% (v/v) glycerol, 0.1 M HEPES pH 7.5. Prior to data collection, crystals were cryo-protected in the mother liquor and flash-cooled by plunging them into liquid nitrogen. X-ray diffraction data to 1.8 Å were collected at the ID30B beamline of the European Synchrotron Radiation Facility in Grenoble, France (ESRF). Image indexing, integration, and data scaling were processed with XDS package (35,36) and programs of the CCP4 suite (37). The Tfu-FNO structure was initially solved by molecular replacement method with Phaser (38) using the coordinates of FNO from *A. fulgidus* [PDB ID code 1JAY (20)] which shares 40% sequence identity with Tfu-FNO as a starting model devoid of all ligands and water molecules. Manual model correction and structure analysis was carried out with Coot (39) whereas alternating cycles of refinement was performed with Refmac5 (40). Figures were generated by using UCSF Chimera (41). Atomic coordinates and structure factors were deposited in the Protein Data Bank under the accession code 5N2I. Detailed data processing and refinement statistics are available in Table 3.

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions

HK and MWF conceived the study and designed the experiments. HK performed the cloning, expression, purification, and biochemical characterization of the Tfu-FNO wild-type and variants. QTN performed the crystallization and solved the structure of Tfu-FNO. AM and CB supervised this part of the study. HK and QTN drafted the manuscript. All authors contributed to analyzing the data and writing the paper.

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Table 1 Steady-state kinetic parameters for wild-type and mutant Tfu-FNOs using NADH and NADPH as substrate. n.d., not determined.

Tfu-FNO variant	NADH			NADPH			$k_{cat}/K_{m,\ NADPH}/k_{cat}/K_{m,\ NADH}$
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\ s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\ s^{-1}$)	
Wild-type	14 ± 4.2	2.2 ± 0.4	160	7.3 ± 1.0	3.3 ± 0.1	450	2800
R51A	8.6 ± 0.9	3.2 ± 0.2	370	>180	>1.6	6.2	17
R51V	8.7 ± 1.1	3.4 ± 0.2	290	>180	>1.3	9.3	32
R55A	7.0 ± 0.8	3.0 ± 0.2	420	29 ± 4.0	8.8 ± 0.3	300	710
R55N	6.3 ± 3.6	2.8 ± 0.7	440	>500	n.d.		
R55S	4.4 ± 1.3	3.5 ± 0.4	790	170 ± 38	6.9 ± 0.7	41	52
R55V	9.6 ± 1.4	3.2 ± 0.2	330	49 ± 7.2	n.d.		
S50E	3.2 ± 1.0	2.7 ± 0.3	840	>500	n.d.		
S50Q	8.2 ± 2.7	4.2 ± 0.6	510	>500	n.d.		
T28A	5.0 ± 0.6	2.6 ± 0.1	520	19 ± 2.6	14 ± 0.5	720	1400
R51ER55A	10 ± 2.7	1.6 ± 0.2	160	>500	n.d.		
R51ER55N	6.5 ± 1.3	2.7 ± 0.2	420	>500	n.d.		
R51ER55S	32 ± 1.9	4.9 ± 0.2	150	>500	n.d.		
R51VR55V	10 ± 1.4	2.8 ± 0.2	280	>500	n.d.		
S50ER55A	20 ± 9.2	2.3 ± 0.7	120	>500	n.d.		
S50ER55V	9.8 ± 2.3	1.8 ± 0.2	180	>500	n.d.		
T28AR51V	12 ± 2.2	2.7 ± 0.3	230	>500	n.d.		
T28AR55A	5.4 ± 1.5	2.5 ± 0.3	460	93 ± 29	3.3 ± 0.4	3.5	8
T28AR51VR55V	12 ± 2.2	3.3 ± 0.3	280	>500	n.d.		

Table 2 Primers used in this study. Sites of mutations are marked with oligonucleotides in bold, whereas restriction sites are in bold, italic.

fno genes	Forward primers (5′–3′)	Reverse primers (5′–3′)
FNO WT	TGC***CATATG***TCGATTGCCGTGCTG TCG	***AAGCTT***TAGATGTCGGTGATGCGGATAC
FNO_R51M	TGATTCTCGGTTCG**ATG**AGCGCGGAGCGGG	CCCGCTCCGCGCT**CAT**CGAACCGAGAATCA
FNO_S50Q	GCACGAGGTGATTCTCGGT**CAG**CGGAGCGCG	CGCGCTCCG**CTG**ACCGAGAATCACCTCGTGC
FNO_S50E	GCACGAGGTGATTCTCGGT**GAG**CGGAGCGCG	CGCGCTCCG**CTC**ACCGAGAATCACCTCGTGC
FNO_R55S	GGAGCGCGGAG**AGC**GCCCAGGCGGT	ACCGCCTGGGC**GCT**CTCCGCGCTCC
FNO_R55N	GCGGAGCGCGGAG**AAC**GCCCAGGCGGTTG	CAACCGCCTGGGC**GTT**CTCCGCGCTCCGC
FNO_T28A	GTGCTGGGGGGC**GCG**GGTGATCAGG	CCTGATCACC**CGC**GCCCCCCAGCAC
FNO_R51A	GATTCTCGGTTCG**GCG**AGCGCGGAGCGG	CCGCTCCGCGCT**CGC**CGAACCGAGAATC
FNO_R51V	GATTCTCGGTTCG**GTG**AGCGCGGAGCGG	CCGCTCCGCGCT**CAC**CGAACCGAGAATC
FNO_R51E	GATTCTCGGTTCG**GAG**AGCGCGGAGCGG	CCGCTCCGCGCT**CTC**CGAACCGAGAATC
FNO_R55A	GGAGCGCGGAG**GCG**GCCCAGGCGG	CCGCCTGGGC**CGC**CTCCGCGCTCC
FNO_R55V	GGAGCGCGGAG**GTG**GCCCAGGCGG	CCGCCTGGGC**CAC**CTCCGCGCTCC
FNO_R55E	GGAGCGCGGAG**GAG**GCCCAGGCGG	CCGCCTGGGC**CTC**CTCCGCGCTCC
FNO_S50A	ACGAGGTGATTCTCGGT**GCG**CGGAGCG	CGCTCCG**CGC**ACCGAGAATCACCTCGT

Table 3 Data collection and refinement statistics

PDB ID Code	5N2I
Space group	$P2_12_12_1$
Resolution (Å)	1.80
a, *b*, *c* (Å)	82.4, 86.1,136.8
R_{sym}[a,b] (%)	11.2 (99.1)
Completeness[b] (%)	98.6 (90.2)
Unique reflections	89383
Multiplicity[b]	4.5 (2.8)
I/σ[b]	7.9 (0.9)
$CC_{1/2}$[b]	99.4 (24.7)
Number of atoms:	
protein	6594
$NADP^+$/glycerol/water	4 × 48/7 × 6/600
Average B value for all atoms ($Å^2$)	25.0
R_{cryst}[b,c] (%)	16.5 (34.1)
R_{free}[b,c] (%)	21.4 (35.7)
Rms bond length (Å)	0.019
Rms bond angles (°)	2.02
Ramachandran outliers	0

[a] $R_{sym} = \sum|I_i - <I>|/\sum I_i$, where I_i is the intensity of i^{th} observation and <I> is the mean intensity of the reflection.
[b] Values in parentheses are for reflections in the highest resolution shell.
[c] $R_{cryst} = \sum|F_{obs} - F_{calc}|/\sum|F_{obs}|$ where F_{obs} and F_{calc} are the observed and calculated structure factor amplitudes, respectively. R_{cryst} and R_{free} were calculated using the working and test sets, respectively.

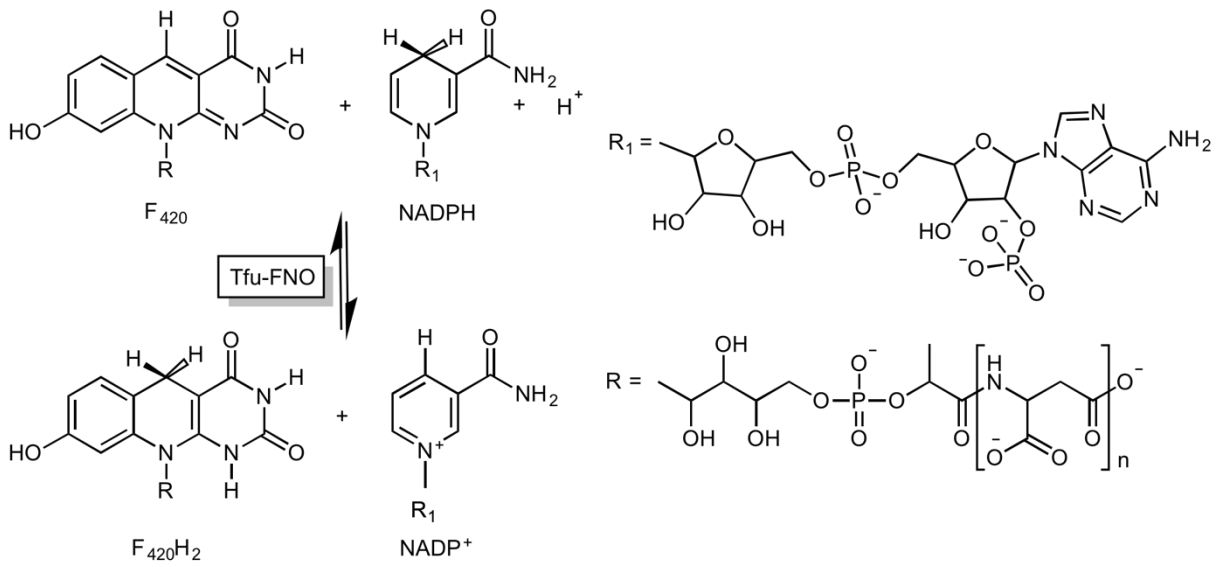


Figure 1 The reversible reaction catalyzed by F_{420}:NADPH oxidoreductase. The number of glutamate residues attached to the phospholactyl moiety may vary (n = 2–8 in case of *Mycobacterium smegmatis*).

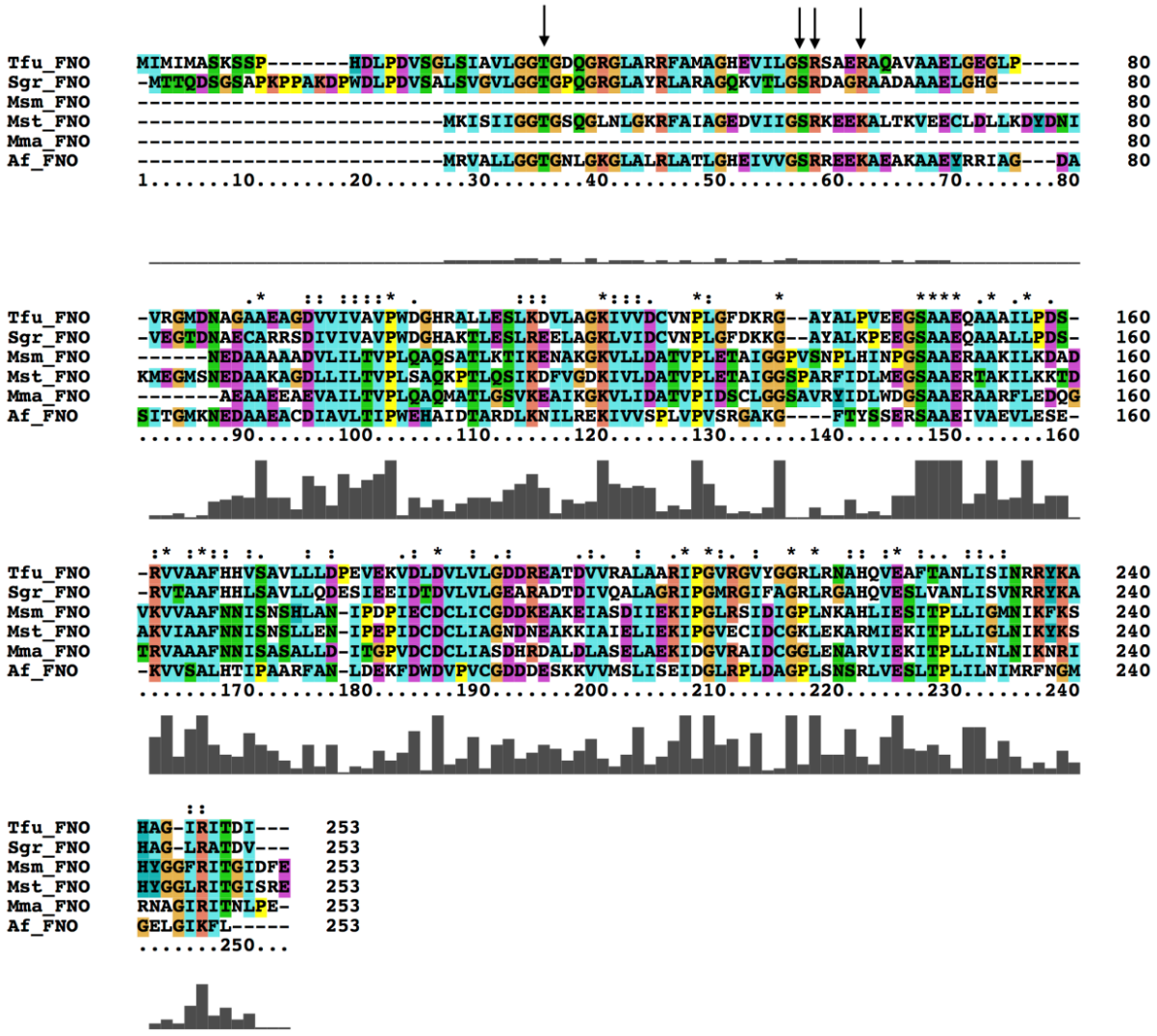


Figure 2 Multiple sequence alignment of selected FNOs from *Thermobifida fusca* (Tfu_FNO), *Archaeoglobus fulgidus* (Af_FNO), *Methanothermobacter marburgensis* (Mma_FNO), *Methanobrevibacter smithii* (Msm_FNO), *Methanosphaera stadtmanae* (Mst_FNO), and *Streptomyces griseus* (Sgr_FNO). The figure was generated with Clustal X2.1. Residues involved in binding the 2′-phosphate group of $NADP^+$ are indicated with an arrow.

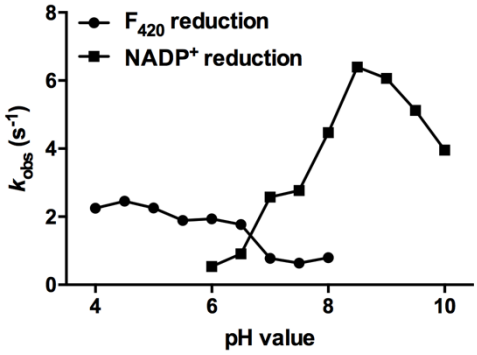


Figure 3 pH optimum for the Tfu-FNO-catalyzed F_{420} reduction using NADPH (dots) or the $NADP^+$ reduction using $F_{420}H_2$ (squares) at 24 °C. k_{obs} (s^{-1}) for the $NADP^+$ reduction (pH optima 8–10) is almost 3-time higher than that for the F_{420} reduction (pH optimum 4–6).

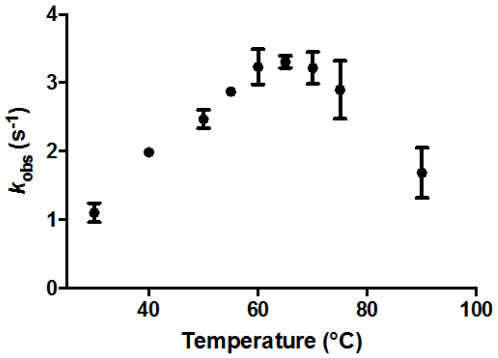
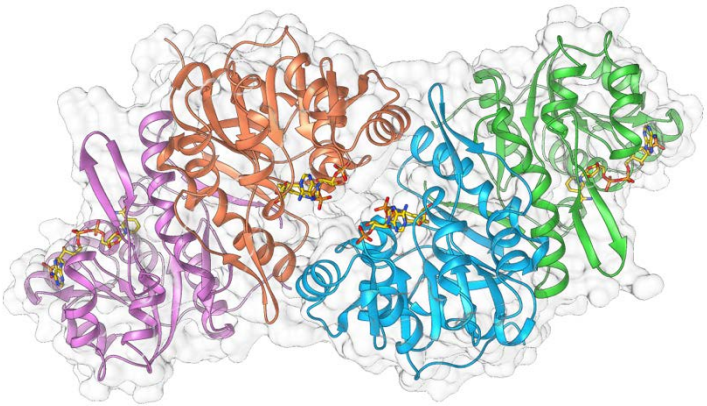
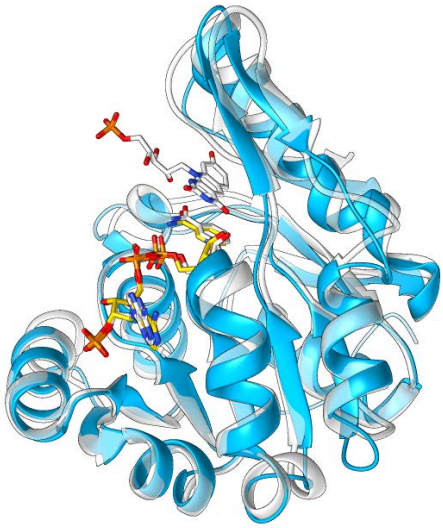


Figure 4 Effect of temperature on Tfu-FNO activity. Reaction mixture of 100 µL contained 1.25 mM NADH, 20 µM F_{420} in 50 mM KPi pH 6.0. The reaction was started by adding 50 nM FNO. The error bars represent standard deviation from two measurements.

A



B



C

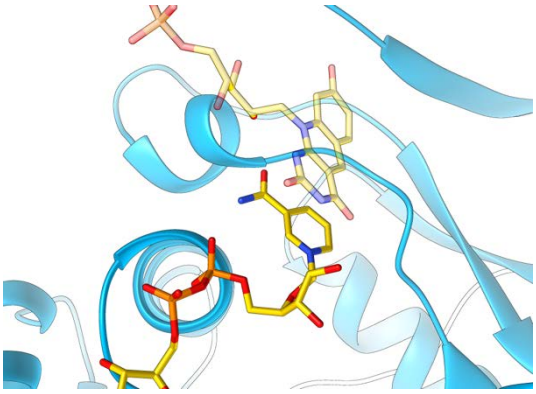


Figure 5 Crystal structure of FNO from *Thermobifida fusca* (**A)** The asymmetric unit of Tfu-FNO crystals contain two dimers AB and CD, colored in coral (monomer A), orchid (monomer B), deep sky blue (monomer C), and green (monomer D), respectively. (**B**) Superposition of the Tfu-FNO monomer C onto the homologous $NADP^+$- and F_{420}-bound Af-FNO monomer [carbon atoms in white, 40% sequence identity, PDB ID 1JAY (20)]. The two structures largely share the same overall topology and the binding pocket architecture, with the nicotinamide rings adopting a similar position in the active site. (**C**) Close-up view of Tfu-FNO binding pocket with a modelled F_{420} molecule (in

shaded colors with carbon atoms in yellow) as a result of superposition as in **B**. The NADP(H) carbon atoms are shown in yellow, oxygen atoms in red, nitrogen atoms in blue, and phosphorous atoms in orange.

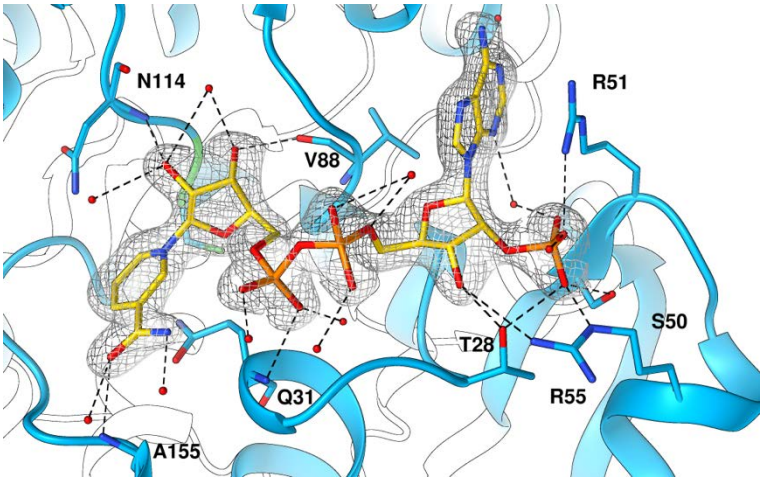


Figure 6 Active site of Tfu-FNO in complex with NADP$^+$. Unbiased $2F_o - F_c$ electron density map calculated at 1.8 Å and contoured at 1.0 σ are drawn as grey chicken-wire. Potential hydrogen bonds are depicted with dashed lines and water molecules as red spheres. Residues in direct contact with NADP$^+$ are labeled. The orientation of the molecule is approximately 180° clockwise rotated along an axis perpendicular to the plane of the paper with respect to that in Figure 5. Color coding for atoms is as in Figure 5.

Isolation and characterization of a thermostable F_{420}:NADPH oxidoreductase from *Thermobifida fusca*

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